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Award Number: DAMD17-00-1-0310

TITLE: Involvement of Tyrosine Phosphatases in Insulin Signaling and Apoptosis in Breast Cancer

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REPORT DATE: June 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Annual (1 Jun 01 - 31 May 02)

4. TITLE AND SUBTITLE
Involvement of Tyrosine Phosphatases in Insulin Signaling and Apoptosis in Breast Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0310

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Our preliminary studies demonstrated that increased intracellular calcium activates calpain to induce partial proteolysis and cytoplasmic translocation of PTP1B, a tyrosine phosphatase proposed to regulate signaling by insulin, IGF-1 and other cytokines. Cytoplasmic translocation of PTP1B results in a distinct pattern of protein tyrosine phosphorylation and these events may regulate cell growth or apoptosis by reducing activated growth factor signaling. In this scheme, calcium-mediated apoptosis and growth inhibition may be directed through mobilization of PTP1B. To investigate this possibility we cloned and expressed a cytoplasmic form of PTP1B (PTP400) in MCF-7 breast cancer cells. PTP400 expression suppressed insulin signaling, reduced cell growth and induced apoptosis in MCF-7 cells. Interestingly, although signaling and growth inhibition by PTP400 were dependent on phosphatase activity, apoptosis was not. Further, PTP400 complexes with Jak2, a tyrosine kinase involved in signaling by multiple cytokines. Together, these results suggest that PTP400 regulates cytokine and growth factor signaling and mediates apoptosis of breast cancer cells. We are currently testing the activity of PTP400 in other breast cancer cells and mapping the PTP400 domain necessary for apoptosis in the hope of identifying novel approaches to therapy for breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer	22
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Tyrosine phosphorylation is regulated by the controlled activation of protein tyrosine kinases and phosphatases (PTP). While kinase activation occurs primarily through auto- and trans-phosphorylation, phosphatase activation is controlled by a variety of mechanisms. One example of distinct phosphatase regulation is PTP1B, a highly expressed tyrosine phosphatase that exists as an endoplasmic reticulum (ER)- associated protein. The hydrophobic C-terminal domain of PTP1B directs its association with the outer leafet of the ER membrane such that its catalytic activity extents into the cytosolic compartment. Importantly, PTP1B has been shown to dephosphorylate several proteins including those that regulate hormone (insulin), growth factor (EGF, IGF) and cytokine (prolactin, leptin) activity. Therefore regulation of PTP1B activity may play an important role in tumors dependent on these signaling entities or in metabolic disorders such as diabetes. Previous studies demonstrated that PTP1B activity was controlled by its phosphorylation and limited proteolysis. Activation of the Ca+2 sensitive protease calpain by increased cytosolic calcium results in limited proteolysis of PTP1B through cleavage of its hydrophobic c-terminal tail, releasing PTP1B enzymatic activity into the cytosol where signaling intermediates can be dephosphorylated. Therefore, PTP1B proteolysis may provide a novel mechanism for phosphatase activation and regulation of growth factor and cytokine signaling. Since breast cancer cell growth, survival and metastasis are largely controlled by tyrosine phosphorylation-mediated signaling pathways, PTP1B may play a role in their regulation. Alterations in PTP1B localization between the ER and cytoplasm may underlie regulation of multiple tyrosine phosphorylation-dependent pathways. To investigate this potential, the affects of expression of a cytosolic PTP1B which mimics the calpain-cleaved and activated form of this phosphatase (tPTP1B) were examined in MCF-7 cells. Since initial studies suggested that transient expression of tPTP1B induced apoptosis of MCF-7 cells, several vectors were employed to evaluate the mechanism of tPTP1B action on signaling pathways and apoptosis of MCF-7 cells.

BODY

Specific aim 1. To examine the effect of cytoplasmic PTP1B expression on apoptotic sensitivity of breast carcinoma cells.

Four tasks were assigned to accomplish this specific aim. These include the completed subcloning of PTP1B into the pcDNA3 vector with an engineered FLAG tag for simple western identification. Cells were to be transiently transfected and monitored by western blot. Optimal expression conditions were to be assessed. Apoptotic sensitivity was also to be tested by cotransfection with a reporter construct (β-galactosidase). These experiments were to be performed in MCF-7 and compared to SKBr-3 cells.

Most of these tasks were completed and reported in the previous year but several modifications were made due to the limitations of this particular expression system. We initially demonstrated pCMV-Flag-tagged tPTP1B (PTP1B/400) in MCF-7 cells and showed that coexpression with a reporter gene resulted in greater apoptotic effects than empty vector alone (figures 1 and 2). However, apoptotic sensitization did not appear to be totally dependent on PTP1B phosphatase activity since phosphatase dead PTP1B mutants had similar affects. This activity was unexpected and needed to be confirmed by alternate approaches. Therefore, we subcloned the cytoplasmic form of PTP1B (PTP400) into additional expression vectors to obtain stable transfectants or inducible expression of this gene and phosphatase –dead mutants.

We were never able to obtain stable transfectants using the pCMV expression plasmid. Therefore, we analyzed cellular responses to transient transfection using several a FLAG-tagged PTP400 (pCMV-3X-Flag-10; Sigma), a GFP-retroviral expression vector (pLEGFP-C1; Clontech), three tet repressor expression constructs for tet-regulated PTP400 expression (pEC1214A; in-house construct provided by Dr. Xu, Dept. of Molecular Oncology, pcDNA4/TO and pcDNA4/TO/myc-His; Invitrogen). We have subjected cells to G418 selection but have not yet recovered stable transfectants. The data obtained from several studies have provided a potential reason for limited recovery of PTP400 expressing stable clones. It appears that prolonged expression of cytoplasmic PTP400 induced apoptosis that is not dependent on its phosphatase activity. These results suggest that a domain or subdomain of PTP1B induces apoptosis through a phosphatase-independent mechanism. We are now identifying the domain responsible for this activity by deletion analysis.

Specific aim 2. To examine whether expression cytoplasmic PTP1B alters insulin (or IGF-1) signal transduction and survival pathways in breast carcinoma cells and whether HER2 expression alters PTP1B-mediated apoptotic sensitization.

To complete these studies 5 tasks were proposed. 1st we were to transfect cells and measure PTP400 expression using anti-FLAG immunoblotting. As shown in figure 1, PTP400 was detected by this procedure. However, we were unable to obtain stable transfectants in MCF-7 cells. Therefore we subcloned the PTP400 w/t and mutant variants (D181A, C215S) into the tet-repressive vector pEC1214A. Cells were transfected with FUGENE and 2 μg of DNA. Cells cultured in the presence or absence of tetracycline (to modulate PTP400 expression) were examined for PTP400 expression 48 hours after transfection. As shown in figure 3, cells expressed PTP400 in the absence but not presence of tet. G418 resistant cells were selected in the presence of tet and at least 5 clones from each transfectant were analyzed for PTP400 in the absence and presence of tet. Unfortunately stable integration of the pEC1214A vector resulted in minimal control of PTP400 expression and these clones were of little use for these experiments. Therefore, additional tet repressive vectors (2 plasmid T-Rex system from Invitrogen) are being tested for their ability to provide efficient and inducible expression of PTP400. Task 1 and 2 of this aim were completed but will require additional studies to provide definitive analysis of PTP400 function in breast cancer cells.

To address tasks 3 and 4, we used a new electroporation system for transient transfection that results in 60 to 80 % transfection efficiency (AMAXA Technology). We also employed standard transfection methodology but sorted GFP positive cells before analysis.

To address task 3, PTP400 w/t and 2 mutants (D181A, C215S) were subcloned into a GFP-retroviral vector (pLEGFP-C1; Clontech) so that PTP400 was expressed as a GFP fusion protein with GFP at the N-terminus. MCF-7 cells were transfected into 10^6 cells with 5-10 μg of DNA (FUGENE) and after 48 hours GFP (+) cells were sorted by FACS in our Institutional core facility (MD Anderson Cancer Center). One hundred thousand cells were obtained for each construct and these cells were placed in culture overnight before analysis of GFP-PTP400 expression by fluorescent microscopy and immunoblotting. As shown in figure 4, GFP-PTP was expressed throughout the cytoplasm.

Cells were maintained for 4 days and the original number of plated cells was noted. Four days after transfection and selection for GFP (+) cells, cell counts were performed. The absolute cell numbers at the beginning and after 4 days in culture are shown in figure 5 (top). Fold-increase in cell number in various transfectants is also shown (bottom). The results demonstrate that PTP400 with wild-type phosphatase function suppressed MCF-7 cell growth by ~ 40 %. The substrate-trapping PTP400 variant (which functions to suppress turnover of phosphotyrosine on PTP1B substrates) stimulated cell growth by ~ 50 %. The C215S mutant had modest stimulatory effects on MCF-7 cell growth (~ 7 %). These results suggest that PTP400 modulates MCF-7 cell growth and protection of PTP400 substrates increase cell proliferation. These data suggest that the cytoplasmic form of PTP1B functions to regulate cell growth. The mechanism is as yet unknown.

Cells were maintained in culture for 7 days and we noted that GFP-PTP400 (+) cells were largely detached from the culture dish. GFP expression alone did not alter cell adhesion. Detached cells were removed and examined for markers of apoptosis (these studies are still ongoing). Remaining cells were subjected to fluorescent microscopy. As shown in figure 6, MCF-7 cells expressing PTP400 (wild-type and mutants) were not recovered, suggesting that long-term expression of PTP400 results in cellular detachment and anoikis (detachment-induced apoptosis). Note that GFP expression alone does not induce this change in cellular attachment. These experiments suggest that PTP400 induces changes in cell viability that are independent of phosphatase activity. We are confirming these results and examining PTP400 domains responsible for this activity. These results were not anticipated and suggest that PTP400 has at least 2 anti-tumor activities. PTP400 suppresses growth, possibly through its effects on tyrosine phosphorylation, but cell attachment is in peril by continual expression of PTP400. This latter activity is not solely dependent on tyrosine dephosphorylation. These results may explain the difficulty associated with establishment of stable PTP400 expressing cells.

To address task 4 and 5 we first monitored the effects of growth factors (insulin, heregulin) and prolactin on tyrosine phosphorylation and Stat5 activation, respectively. As shown in figure 7, insulin and heregulin increased tyrosine phosphorylation in MCF-7 cells but differed in their pattern of phosphotyrosine induction. Prolactin did not induce global changes in tyrosine phosphorylation but increased activation of Stat5, which is known to be regulated through cytokine-receptor mediated activation of Jak2. The effects of PTP400 expression on signaling by each factor are currently being examined. However, initial studies are focused on its affects on insulin signaling which had previously been suggested to be under the control of PTP1B.

The effects of GFP-PTP construct expression on total tyrosine phosphorylation were examined in MCF-7 cells. MCF-7 cells were transfected with various GFP-PTP constructs or GFP alone using FUGENE. Approximately 10 % of the population was transfectable by examination of cells by fluorescent microscopy. Cell lysates were prepared and analyzed for PTP expression and changes in phosphotyrosine content. As shown in figure 8, expression of GFP-PTP was easily detectable after transfection. Importantly modest changes in protein phosphorylation were also noted in cells expressing the wild-type PTP400 construct.

Specific changes were noted in phosphorylation of the 150 and 75kDa proteins. Also, it appears that the substrate trap variant of PTP400 stabilized phosphorylation of similar proteins suggesting these to be major substrates for PTP1B. The identity of these proteins is being examined by immunoprecipitation and immunoblotting of phosphotyrosine in "substrate trapped" molecules from cell lysates. Early studies have indicated a possible role for Jak2 in these complexes (figure 11). Overall, these results demonstrate that GFP-PTP expression results in changes in tyrosine phosphorylation and expression of PTP400 as a GFP fusion protein does not affect its enzymatic activity.

Since expression of PTP400 appears to induce growth inhibition and subsequent apoptosis of MCF-7 cells we needed to analyze signaling after transient transfection. We are also testing a new tet-switch expression system to further investigate these processes (T- REx, Invitrogen). To advance this project we employed a new gene transfer mechanism that utilizes electroporation with proprietary solutions that provide efficient gene transfer. We screened and found that solution V from AMAXA technology provides a very efficient means of transferring genes into MCF-7 cells. Using this approach, we achieved ~ 60 % expression efficiency when detected by fluorescence microscopy. As shown in figure 9, we were able to express GFP-PTP400 and variants in MCF-7 cells. Changes in tyrosine phosphorylation were monitored in transfected cells treated with insulin or left untreated. As previously noted, wild-type PTP400 reduced tyrosine phosphorylation in control and insulin-treated cells. At least 3 proteins (#1-3) were regulated by PTP400 expression. Again, the substrate-trap variant D181A, which functions as a dominant-negative of PTP1B, increased tyrosine phosphorylation of the same proteins suppressed by wild-type PTP400. These results demonstrate a consistent change in tyrosine phosphoproteins and these target proteins may play a role in the control of MCF-7 cell growth. These experiments are to be conducted in HER2 expressing cells, as reported in Task 5 of this specific aim.

To determine the effect of PTP400 on signal transduction, cells were transfected with the pLEGFP vector using the AMAXA system and analyzed for tyrosine phosphocontent, and specific downstream targets previously suggested as PTP1B targets. As shown in figure 10, PTP400 suppressed tyrosine phosphorylation of at least 4 proteins (# 1,2,3,5) and #4 was suppressed by PTP400 in insulin-treated cells. The C215S variant partially suppressed dephosphorylation of these 5 proteins, an activity shared with the D181A variant. Interestingly, this variant had a significant effect on phosphorylation of a ~ 150kDa protein, as shown in earlier studies. These results suggest that this phosphoprotein is a major target for PTP400-mediated dephosphorylation. We also monitored downstream signaling events that were previously shown to be regulated by insulin (Akt or PKB) or PTP1B (c-src). Interestingly, Akt activation was not suppressed by expression of PTP400 while the activated form of c-src (Y416) was more highly activated in the presence of PTP400. Other downstream targets are now being examined but these initial experiments suggest that insulin-mediated signaling is not highly sensitive to regulation by PTP400. Other signaling pathways may be more sensitive to PTP400-mediated dephosphorylation.

The tyrosine kinase Jak2 has recently been shown to complex with PTP1B and regulate signaling initiated by specific cytokines, such as those that regulate lipid and carbohydrate metabolism (i.e. leptin). To determine if Jak2 activation is affected by PTP400 expression, transfected cells were analyzed for changes in phosphorylation of this kinase. The sites recognized by the p-Jak2 antibody represent tyrosine site that correlate with Jak2 activation (Y1007, Y1008). Jak2 phosphorylation was not affected by expression of PTP400 but expression of the dominant-negative PTP400 (D181A) enhanced Jak2 phosphorylation in MCF-7 cells. These results confirm studies of PTP1B function in other cell types and suggest that Jak2 functions as a target for PTP400 regulation in breast cancer cells as well. This is significant since this kinase controls phosphorylation of transcription factors that support cellular transformation of breast cancer cells (Stat). We are currently analyzing the effect of PTP400 and variant expression on Jak2-dependent cytokine initiated signaling in MCF-7 and other breast cancer cells.

We have largely completed the tasks involved in execution of this grant proposal. We are continuing these studies and hope to report these results in several publications that cite the support of the Department of Defense. We are also nearing completion of stable transfectants that regulate PTP400 expression by tetracycline. These studies as well as studies in HER2 expressing breast cancer cells and signaling studies are to be completed by the end of the year. We are also confirming substrate utilization by PTP400 using immunoprecipitation and immunoblotting analysis of "substrate-trapped" PTP400 molecules. If necessary unknown substrates will be investigated through mass spec analysis of peptides derived from these phosphoproteins.

KEY RESEARCH ACCOMPLISHMENTS

1. Variant PTP1B constructs (PTP400) with mutations in the catalytic domain that affect phosphatase activity (C215S) or substrate turnover (D181A) were constructed and used to define the role of phosphatase activity in the biological actions of cytoplasmic PTP1B.
2. Several proteins were suggested as potential substrates of this phosphatase. PTP400, which mimics a form of PTP1B found in calcium-ionophore treated cells, induced breast cancer cell growth inhibition while the substrate trap form of this protein stimulated cell growth. Long term expression of PTP400 induced cell detachment and resulted in death of breast cancer cells through anoikis.
3. GFP-PTP400 fusion protein was detected primarily in the cytoplasm of MCF-7 cells.
4. PTP400 altered tyrosine phosphorylation in MCF-7 cells and modestly suppressed insulin-mediated tyrosine phosphorylation. PTP400 did not suppress Akt activation but did alter the activation state of c-src and Jak2.
5. We have subcloned the PTP400 gene into a new tet-repressible vector (T-REx) to assist in confirming these results and to identify the mechanism of cell growth inhibition, apoptosis and signal inhibition by PTP400.

REPORTABLE OUTCOMES

We have recently presented some of these data at the Era of Hope Department of Defense meeting in Orlando, Florida (September 25-28) [Abstract # P39-3]. A manuscript that details the result of these studies is to be submitted by the end of the year. A graduate student involved in this research effort (Ms. Sharon Beresford) received her doctorate this year. Vectors and cell lines used in this study may be useful in conducted animal studies for breast cancer and as models for diabetes.

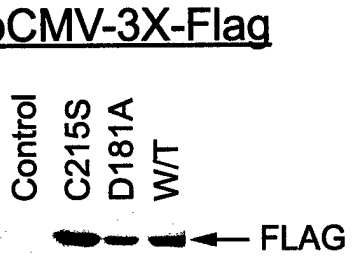
CONCLUSIONS

These data support a role for PTP400 phosphatase in both breast tumor growth and survival. These PTP400 activities were inferred in studies of calcium-ionophore treated cells and confirmed by the current study. Most interestingly, we detected activities of PTP400 that were dependent on its phosphatase activity (growth inhibition, signal regulation) and those that were independent of its enzymatic activity (detachment, anoikis, apoptosis). These observations support my hypothesis of a role for this phosphatase in both control of breast tumor growth and survival as well as a regulator of metabolic disorders such as diabetes. These results support additional investigation of the role of this PTP in breast cancer in women at low risk for this disease (Hispanic, American Indian) but high risk for diabetes. Further, exploiting these activities with PTP400 vectors or isolated domains may lead to better therapy for both breast cancer and metabolic disorders.

REFERENCES

N/A

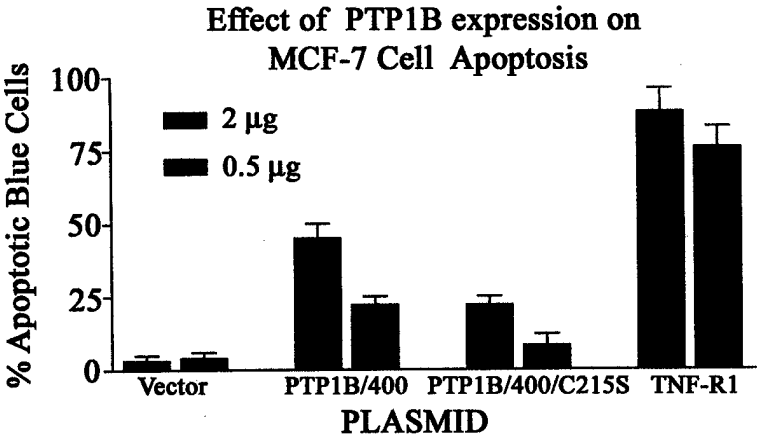
Figure 1.



MCF-7 cells were transfected with 2 µg of pCMV-3X-FLAG (FUGENE) and analyzed for PTP400 expression by anti-FLAG immunoblotting.

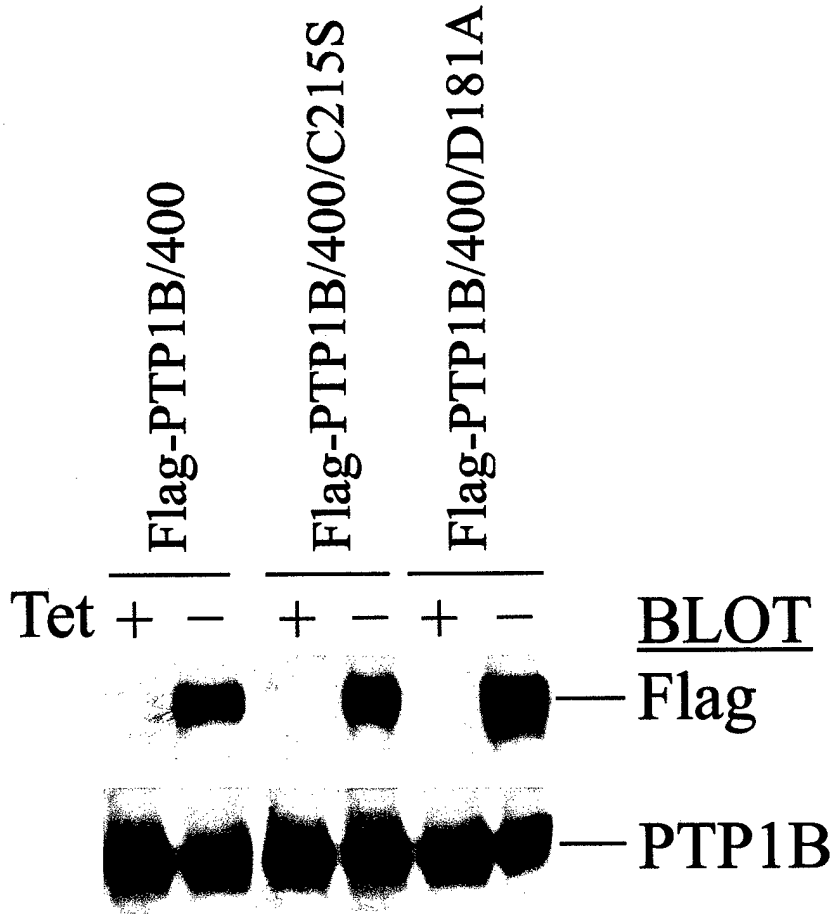
Although PTP400 expression was detected, tyrosine phosphorylation was not affected (not shown). Stable transfectants were not recovered after multiple transfections

Figure 2.



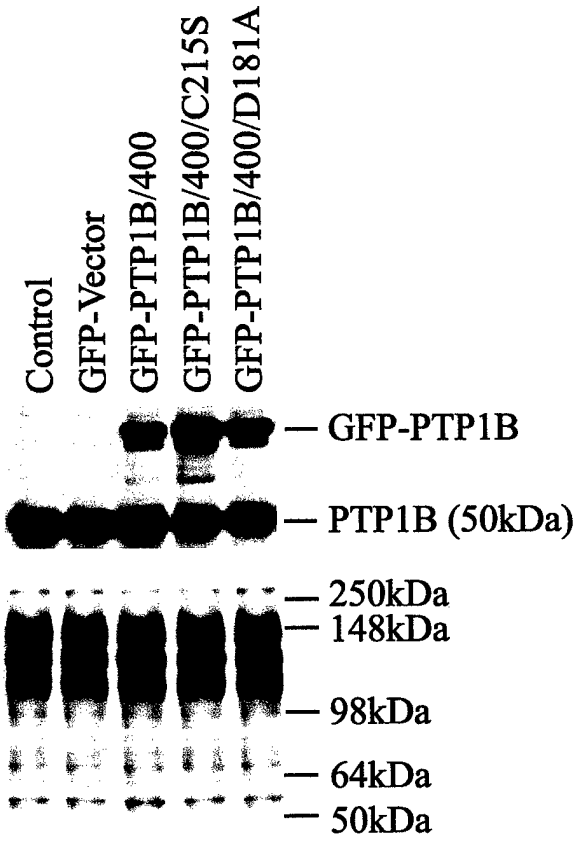
MCF-7 cells were cotransfected with pcDNA3 plasmid (as noted) and 0.5 µg β-galactosidase. After 24 hours, cells were fixed and stained with X-gal and blue versus apoptotic blue cells were scored by microscopic analysis. The results suggest increased apoptosis in cells expressing phosphatase active PTP1B. TNF receptor 1 expression plasmid was used as a positive control

Figure 3.



MCF-7 cells were transfected with pEC1214A (tet-repressive vector) constructs expressing tPTP1B variants as described above. Lysates were immuno-blotted with anti-Flag or PTP1B. Transfections were performed in the presence or absence of tet as shown. Stable variants (grown and selected in the presence of tet) are currently being screened.

Figure 4.



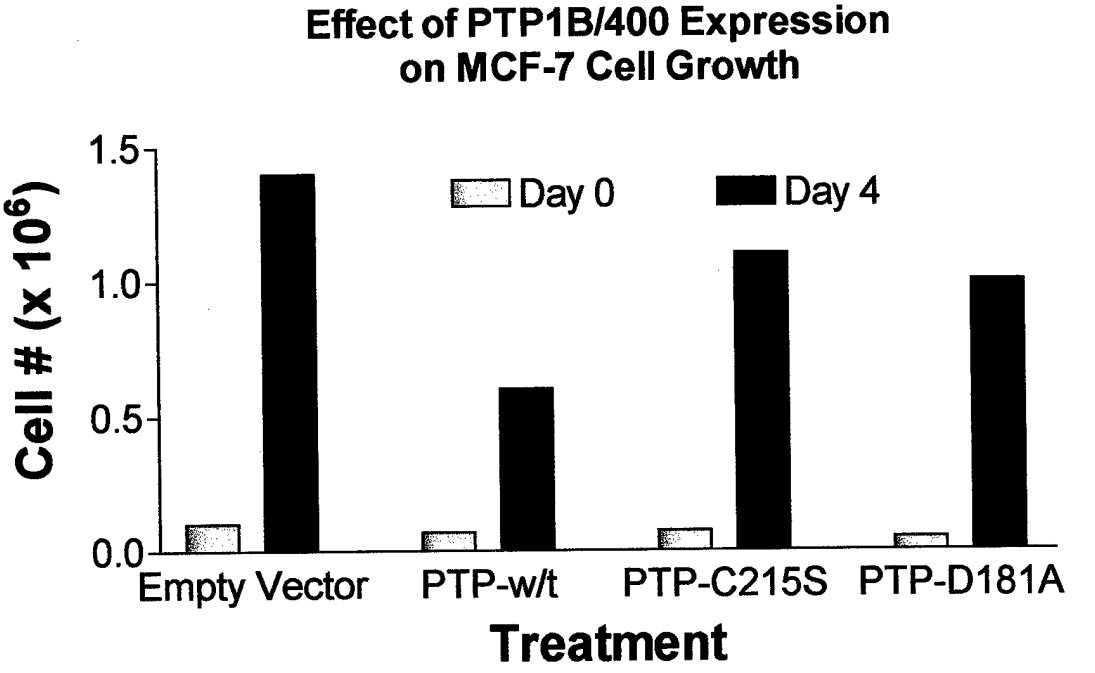
PTP1B truncated at 400 aa was subcloned into the Sal 1/BamH1 site of Clonetech vector . pLEGFP-C1 MCF-7 cells were transfected with 5 µg DNA (FuGENE) and lysates were analyzed for PTP1B expression by immunoblot.

After 48 hours cells were analyzed for GFP-PTP1B expression by fluorescent microscopy. GFP-PTP1B truncated at the ER localization domain results in expression of a predominantly cytoplasmic protein.

Phase

Fluorescence

Figure 5.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Day 4
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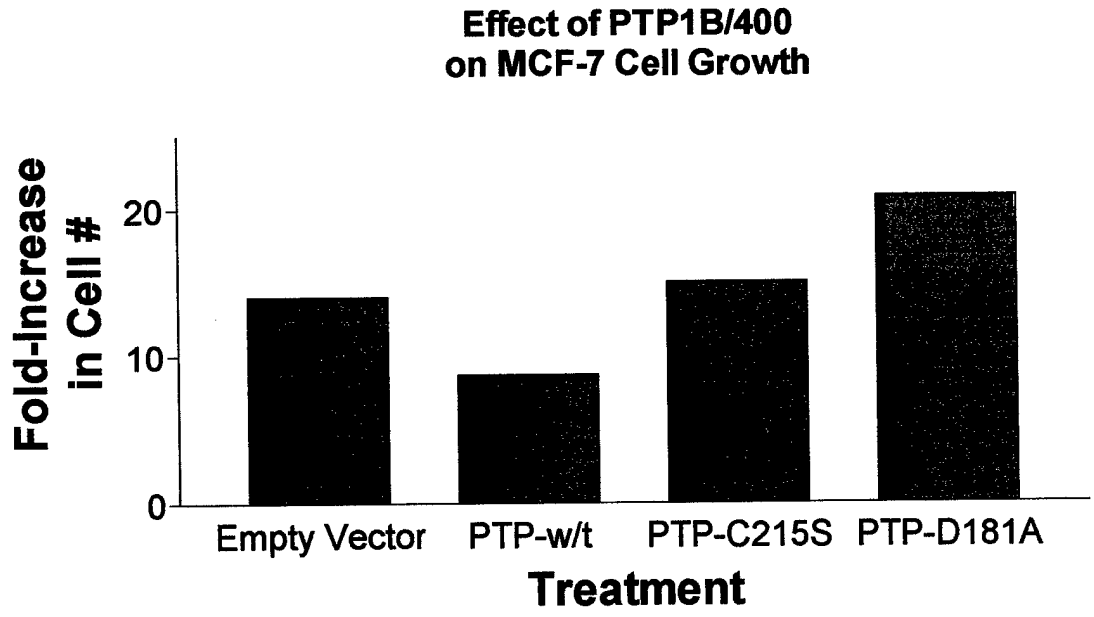
PTP-C215S
PTP-D181A
Treatment


Effect of PTP1B/400
on MCF-7 Cell Growth
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in Cell #
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10
0
Empty Vector
PTP-w/t
PTP-C215S
PTP-D181A
Treatment

Figure 6.

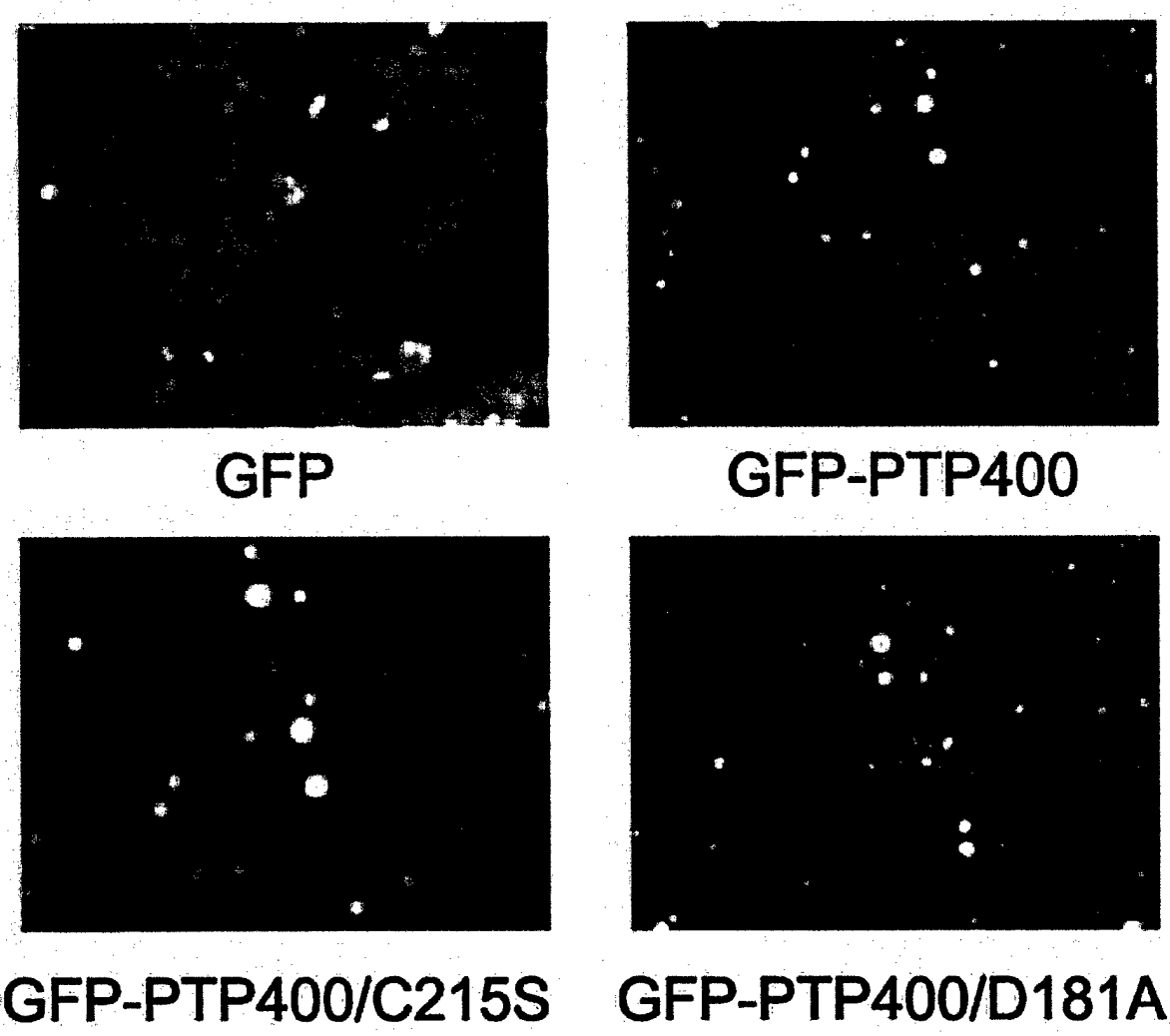
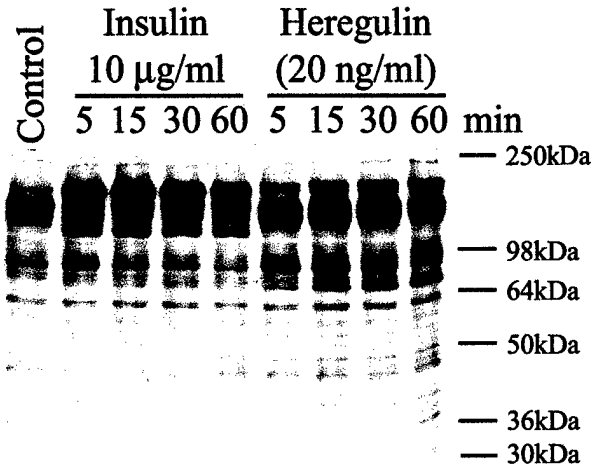
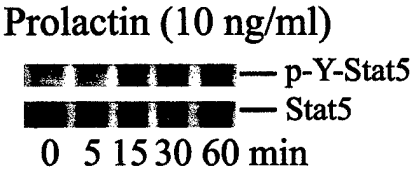


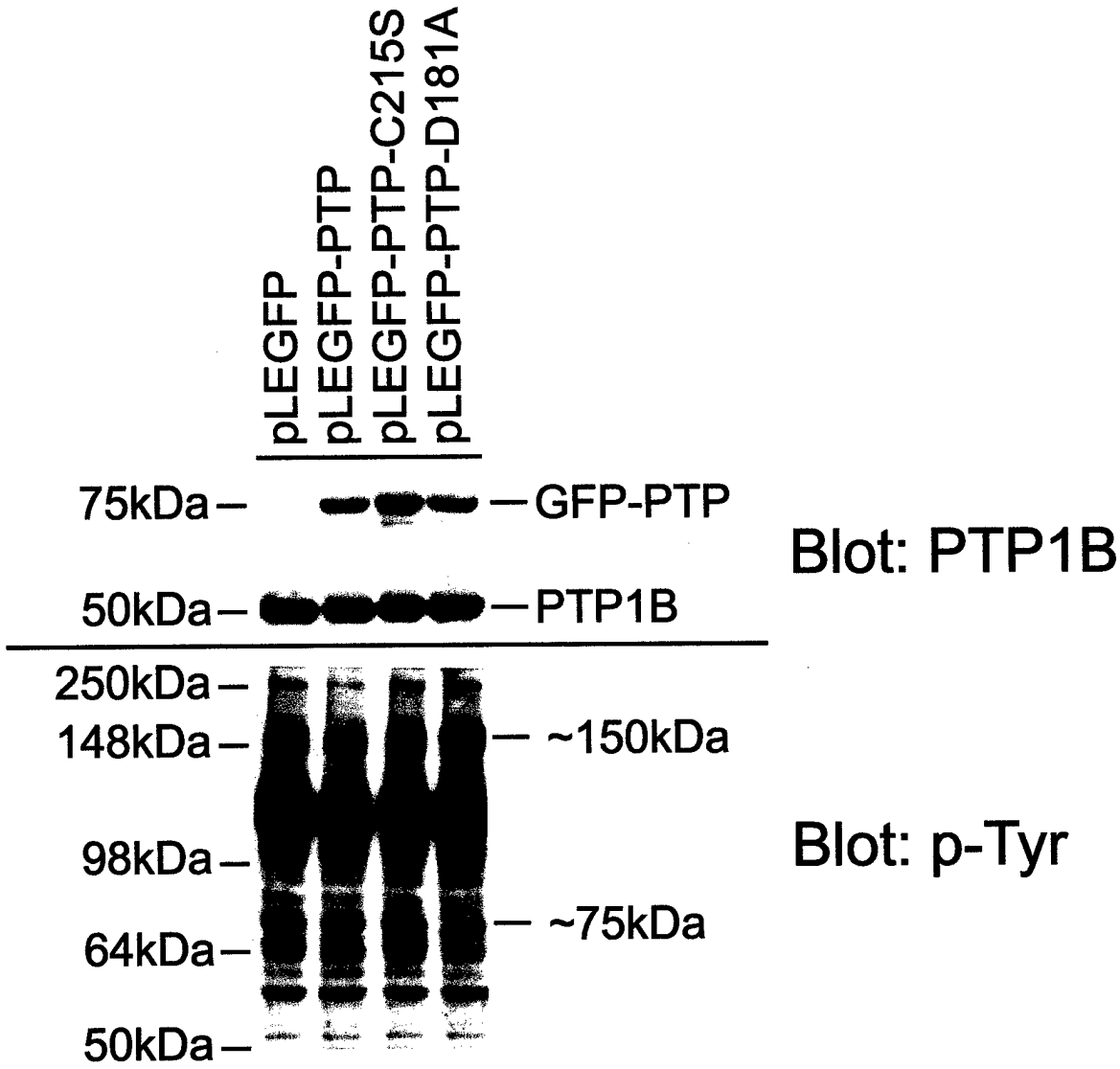
Figure 7.





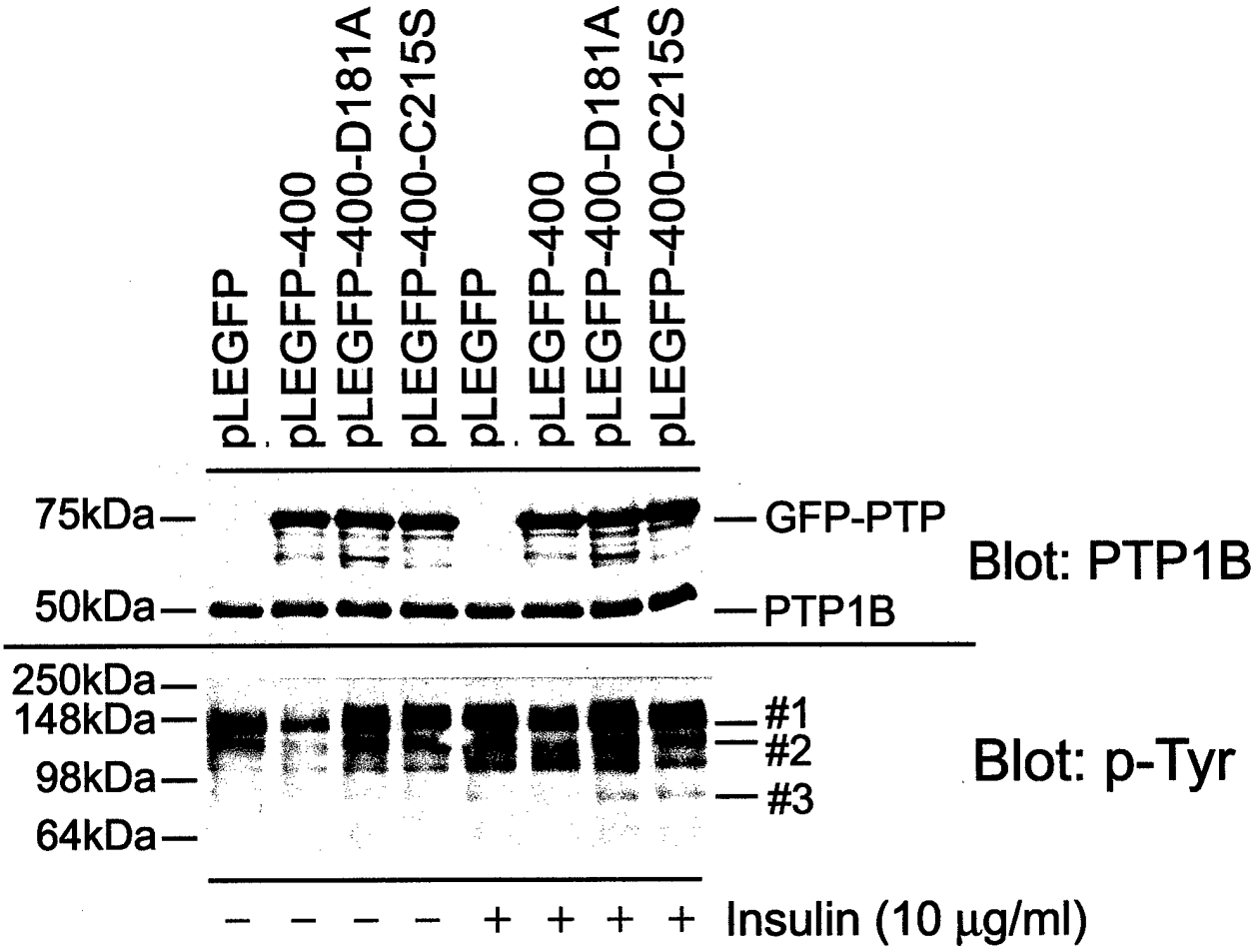
MCF-7 cells were treated with growth factor or cytokine for the interval noted and tyrosine phosphorylation (top) or Stat5 tyrosine phosphorylation (bottom were assessed. Treatment of MCF-7 cells induced non-overlapping patterns of tyrosine phosphorylation. The effect of tPTP1B expression on these events is currently being evaluated.

Figure 8.



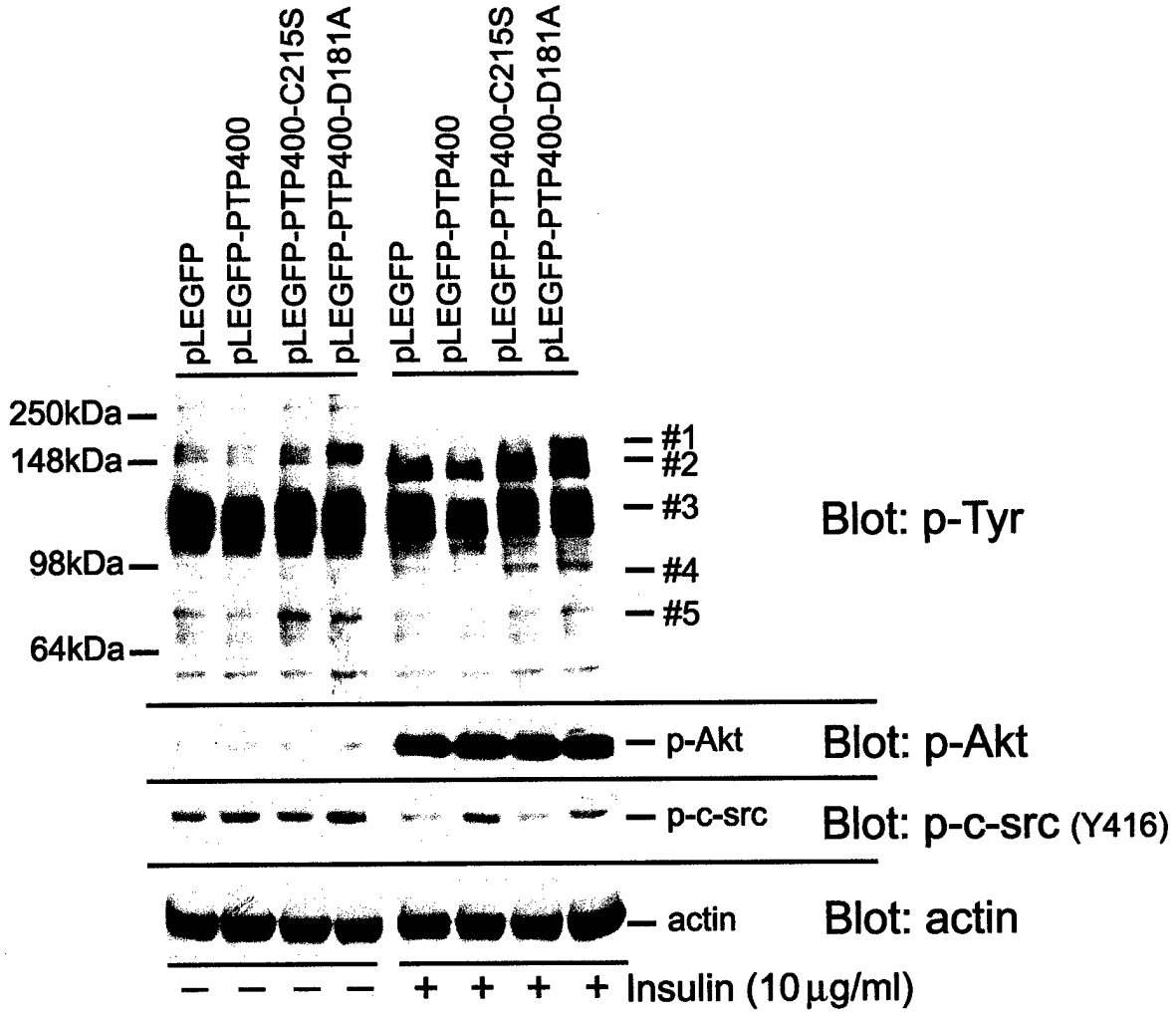
MCF-7 cells were transfected with the indicated vector and 48 hours later cell lysates were analyzed for PTP1B expression (top) and phosphotyrosine content (bottom). Although only 10 % transfection efficiency, modest changes in p-Tyr levels were seen in cells transfected with w/t PTP400 (note 150 and 75kDa phosphoproteins).

Figure 9.



MCF-7 cells were electroporated with the indicated construct using the AMAXA gene transfer system. Transfection effeciency is ~60 % utilizing this system. Twenty-four hours after transfection cells were serum starved overnight and left untreated or stimulated with insulin for 30 min. as noted. Cell lysates were examined for PTP1B expression (top) and phosphotyrosine content (below). At least 3 phosphoproteins were affected by insulin and PTP expression (labeled # 1-3). These results demonstrate that cytoplasmic PTP400 effects bothe endogenous and insulin stimulated protein tyrosine phosphorylation.

Figure 10.



MCF-7 cells transfected with the indicated vector (as described in figure 9) were incubated overnight in complete growth media before shifting cells to serum-free media. Cells were then treated with media alone or insulin as noted for 30 min. Cell lysates were prepared and analyzed for phospho-tyrosine (top), activated Akt or c-src. Protein load was monitored by blotting actin. Wild-type PTP400 expression suppressed both endogenous and insulin stimulated tyrosine phosphorylation but did not affect activation of Akt by insulin. PTP400 expression increased activation of c-src following insulin stimulation. Expression of the substrate-trapping PTP400 variant stabilized phosphorylation of an unknown protein of ~ 150kDa. At least 5 tyrosine phosphoproteins were affected by PTP400 or variant expression.

Figure 11.

MCF-7 cells transfected as desribed in figure 9 were harvested 48 hours after AMAXA system transfection and analyzed for changes in activation of Jak2. As shown, expression of the substrate-trapping PTP400 (D181A) variant enhanced tyrosine phosphorylation of Jak2. These results suggest that PTP1B suppresses Jak2 activation in MCF-7 cells.